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Constitutively active Cullin-RING-Ligases fail to rescue loss of NEDD8 conjugation in *Schizosaccharomyces pombe*

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ABSTRACT

In fission yeast, the only known essential function of Ned8p is the modification of the cullin, Pcu1p, and subsequent Cullin-RING-Ligase (CRL) activation and substrate ubiquitination. We show here that a functional Pcu1p mutant, deleted for its C-terminal autoinhibitory domain, which negates the requirement of neddylation for ligase activity, is unable to rescue the loss of neddylation. These findings suggest that the neddylation of non-cullin substrate(s) are required for *Schizosaccharomyces pombe* viability.

1. Introduction

NEDD8 is a member of the ubiquitin-like family of proteins, and in a manner analogous to ubiquitination, neddylation is achieved via the actions of its cognate E1-like, and E2-like enzymes. Cullin neddylation is enhanced by the E3-like action of the recently identified scaffold protein DCN1, that acts to recruit both UBC12 and cullin proteins [1,2]. Neddylation is dynamic and can be reversed via the metalloprotease activity of the CSN5 subunit of the COP9 signalosome complex [3,4]. Cullin neddylation serves to activate the CRL complex by increasing the affinity of the charged ubiquitin E2 to the ligase complex [5,6], and by bringing about a major conformational change resulting in the displacement of the inhibitory extreme C-terminal domain (ECTD)/winged-helix B (WHB) sub-domain of the cullin [7,8]. Neddylation is, however, not limited to the modification of cullin proteins and regulation of CRL activity, as roles have been reported in modulating transcriptional activity and membrane receptor signalling [9,10].

Mono-neddylation is essential in *Schizosaccharomyces pombe* [11,12]. To date, the only identified substrates in *S. pombe* are Pcu1p, Pcu3p, and Pcu4p, members of the cullin family. Ned8p modification of Pcu1p, the only essential cullin family member, is required for function, as mutation of the acceptor lysine residue results in lethality [11].

Here, using *S. pombe*, we show that Pcu1p mutants lacking their autoinhibitory domain, no longer require neddylation for function in vivo, and yet fail to rescue the lethality of the uba3 conditional mutant. Our results suggest that although cullin neddylation is essential, neddylation is also important in protein regulation outwith this modification.

2. Materials and methods

2.1. Yeast strains and general methods

Fission yeast was maintained on rich medium (YES) or on Pombe minimal medium with glutamate (PMG) with appropriate supplements where selection was necessary. General methods for handling the *S. pombe* strains are as described [13]. Strains used in this study are listed in Table 1. Cadmium chloride was added to a final concentration of 0.5 mM and L-canavanine to that of 2 μg/ml. To induce the expression of nmt1 promoters, cells were grown in PMG media supplemented with 15 μM thiamine to mid-log phase, washed three times and resuspended in PMG lacking thiamine for 18 h of growth to the start of exponential growth. Bioneer diploids were haplodised by growth on media containing 20 μg/ml thiabendazole (TBZ).

2.2. Cell viability test

Wild-type, *uba3–10*, *uba3–10Δdcn1*, *uba3–10Δcsn5*, and *pcu1–2* cells were grown at 36 °C for the indicated time. Cell number

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Table 1
Strains used in this study.

Strains	Genotype	Derivations
"*Wild-type*"	*h⁻ ura4 leu1 ade6*	Our stock
uba3–10	*h⁻ uba3::ura4 leu1 ura4 ade6*	This study
pcu1–13myc	*h⁺ pcu1::13myc KanMX ura4 leu1*	Mundt et al. [18]
pcu1–13mycΔcsn5	*h⁺ pcu1::13myc KanMX csn5::ura leu1*	Mundt et al. [18]
pcu1–13myc uba3–10	*pcu1::13myc KanMX uba3::ura4 leu1ura4 ade6*	This study
Δdcn1 diploid	*h⁺ dcn1::KanMX4 leu1/leu1 ura4/ura4 ade6/ade6*	Bioneer
Δdcn1 G418ʳ	*h⁺ dcn1::KanMX4 leu1 ura4 ade6*	This study
Δdcn1 NATʳ	*h⁺ dcn1::NatMX6 leu1 ura4 ade6*	This study
uba3–10Δcsn5	*uba3::ura4 csn5::ura4*	This study
uba3–10Δdcn1	*uba3::ura4 dcn1::NatMX6*	This study
pcu1–13mycΔdcn1	*h⁺pcu1::13myc KanMX dcn1::NatMX6 leu1 ura4 ade6*	This study
pcu1–13mycΔcsn5Δdcn1	*pcu1::13myc KanMX csn5::ura dcn1::NatMX6 leu1 ade6*	This study
pcu3–13myc	*pcu3::13myc KanMX ura4 leu1*	Zhou et al. [20]
pcu3–13myc Δdcn1	*pcu1::13myc KanMX dcn1::NatMX6 leu1 ade6*	This study
pcu3–13myc uba3–10	*pcu3::13myc KanMX uba3::ura4 leu1 ura4 ade6*	This study
skp1–3	*h⁻ skp1::KanMX leu1 ura4 ade6*	Hermand et al. [21]
pcu1–2	*h⁻ pcu1::ura4 leu1 ade6*	This study

was determined by the counting of cells on a Coulter counter. Subsequently 200 cells were spread on YES plates. The plates were then incubated at the permissive temperature, 25 °C, and the number of colonies was counted after five days.

2.3. Construction of temperature-sensitive (ts) mutant strains

The *uba3–10* and *pcu1–2* mutants were generated by error prone PCR of genomic DNA followed by replacement of the ORF using the method of MacIver et al. [14].

2.4. Plasmid construction

The gateway pDUAL-FFH1c plasmids containing the genomic clones 20/F06 (*S. pombe uba3⁺*, SPAC24H6.12c) and 47/B10 (*S. pombe pcu1⁺*, SPAC17G6.12) was provided by RIKEN BRC, which is participating in the National Bio-Resources Project of the MEXT, Japan [15]. Pcu1 C-terminal deletion mutants were generated with the universal gateway compatible primer (5′) GGGGACAAGTTTGTACAAAAAAGCAGCCTTCATGACTACTTTGAATACC in conjunction with GGGGACCACTTTGTACAAGAAAGCTGGG TACTAATCTATACATTGCTTAA TATC (3′) and GGGGACCACTTTGTACAAGAAAGCTGGGTACTATTTCAATGTTCGGCGAGCG (3′) to generate Pcu1Δ748–767 and Δ720–767, respectively. The Pcu1K713R point mutant was generated via the QuickChange™ site-directed mutagenesis (Stratagene) kit with the oligonucleotides: (5') CGTCCGTATCATGAGGGCTCGCC GAACATTG and CAATGTTCGGCGAGCCCTCATGATACGGACG (3′). All mutations were confirmed by automated DNA sequencing.

Rum1⁺ was cloned from genomic DNA using the gateway compatible primers: (5′) GGGGACAAGTTTGTACAAAAAAGCAGGCTC TCATATGGAACCTTCAACACCACC and GGGGACCACTTTGTACAAGA AAGCTGGGTATCGTAATAAATTGTGCCTG (3′). Following recombination via the Gateway™ reaction, as per manufacturer's instructions (Invitrogen), into the pDONR221 vector, the plasmids were once again combined with pDUAL-FFH1c as per manufacturer's instructions.

2.5. Preparation of total cell extracts

Logarithmically growing cells (10^8) were harvested, and prepared under denaturing conditions with trichloroacetic acid (TCA) [16]. Samples were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blot analysis. MYC and FLAG epitopes were detected with the monoclonal antibodies: anti-MYC (Covance) and anti-FLAG (SIGMA), respectively.

3. Results

3.1. Generation and phenotypic analysis of uba3–10

To further elucidate the in vivo function of neddylation we generated conditionally lethal ts mutants of *uba3⁺* by random mutagenesis followed by chromosomal integration (Fig. 1A). The mutation sites within *uba3⁺* (Table 2) were found to be in evolutionary conserved amino acids. Mapping the mutations onto the determined crystal structure of the UBA3–APPBP1–NEDD8 complex [17] (Fig. 1B) indicated the mutated residues were buried, and would not be involved in direct contact with Ned8p, Uba5p, or Ubc12p. To confirm that the temperature sensitivity of the *uba3–10* strain was solely due to the mutation of *uba3⁺*, we crossed the *uba3–10* strain to its opposite mating type wild-type strain and showed that the temperature sensitivity segregated $2ts^+$:$2ts^-$ (data not shown) and could be rescued by exogenous expression of *uba3⁺* (Fig. 1C).

3.2. Characterisation of the uba3 mutant phenotype

Cultures of early log phase growing *uba3–10* and wild-type cells were shifted from the permissive to the restrictive temperature and sampled every two hours. Cell number of *uba3–10* failed to increase (Fig. 1D), and viability decreased (Fig. 1E).

3.3. Impaired neddylation in the uba3–10 strain

A reduction in Ned8p function would be expected due to the loss of Ned8p conjugation as a consequence of the mutated *uba3* gene. To investigate this, in the absence of any available Ned8p antibody, the *uba3–10* strain was crossed to a myc-tagged *pcu1⁺* allele bearing strain, a known and well-characterised substrate of neddylation. This strain has previously been used to show that under normal conditions, Pcu1p is found in a predominately Ned8p modified state [18] (Fig. 1F). Western blot analysis of the *pcu1⁺–13myc uba3–10* strain lysates prepared under denaturing conditions showed that the level of Pcu1p neddylation was significantly reduced compared to wild-type, although still detectable, at the permissive temperature. Strikingly the level of Pcu1p neddylation was undetectable after only two hours of growth at the restrictive temperature (Fig. 1F), although the strain maintained viability (Fig. 1E). To confirm that the *uba3–10* mutant cells would effect the stabilization of a known CRL1 substrate, Rum1p [11,19], at the restrictive temperature, a plasmid expressing FLAG-tagged

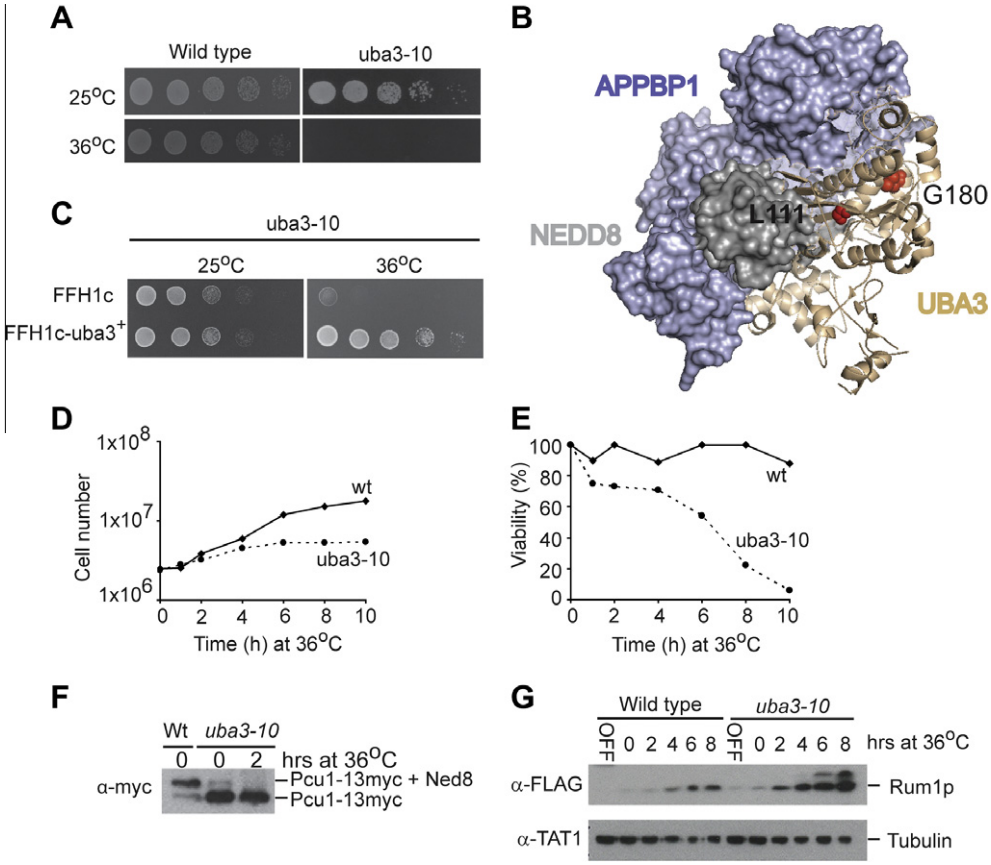

Fig. 1. *uba3–10* cells are temperature-sensitive. (A) Stepwise fivefold serial dilutions of wild-type and *uba3–10* yeasts were spotted onto PMG-Ura plates and incubated at the indicated temperatures for 3–5 days (initial spots contained 1×10^5 cells). (B) Following sequence alignment the equivalent amino acids found mutated in *S. pombe uba3–10* were identified in the human UBA3 protein, and were mapped onto the crystal structure of the UBA3–APPBP1–NEDD8 complex. Images were made using MacPyMOL with (PDB:1YOV). Mutated residues are indicated in red. (C) Stepwise fivefold serial dilutions of the *uba3–10* yeast, integrated with the indicated plasmid, grown on PMG-Ura plates at the indicated temperature (initial spots contained 1×10^5 cells). (D) Wild-type or *uba3–10* yeast was grown in YES and shifted to 36 °C, and the cell number was determined at the indicated times. (E) As (D), but at each time point, 2×200 cells were plated onto YES medium and incubated at 25 °C for 5–7 days. Colonies on each plate were counted and the percentage of viable cells was calculated by normalisation to the colony number of the 0-h samples. (F) *uba3–10* cells containing a tagged allele of *pcu1*$^+$ were grown at 36 °C for the indicated times. Lysates were prepared under denaturing conditions and analysed by SDS-PAGE and immunoblotted with anti-myc antibody. (G) Time-course of wild-type and *uba3–10* cells expressing pDUAL-FFH1c *rum1*$^+$ were shifted to the restrictive temperature for the indicated times. Lysates were prepared and analysed as in (F), but immunoblotted with anti-FLAG antibody.

Table 2
Mutation sites of uba3 ts mutant strain.

Allele	Mutation sites
uba3–10	I107V, G169D

rum1$^+$, was integrated into wild-type and *uba3–10* cells. Expression was allowed to proceed for 18 hours in the absence of thiamine, prior to cultures being shifted from the permissive to the restrictive temperature. Subsequently, samples were taken every two hours for eight hours, and total extracts were prepared under denaturing conditions followed by immunoblotting with anti-FLAG antibody. Inactivation of the Ned8p pathway in the *uba3–10* strain consistent, with previous reports, resulted in a time dependent stabilization of Rum1p (Fig. 1G).

3.4. Levels of Pcu1p neddylation do not directly correlate with viability or sensitivity to stress conditions

Neddylation of Pcu1p is regulated via the E3 ligase function of Dcn1p and the metalloprotease activity of the Csn5p subunit of the COP9 signalsome. Deletion of *csn5*$^+$ (Δ*csn5*) had previously been shown to dramatically enhance neddylation of Pcu1p [3,18]. To investigate the neddylation status of Pcu1p, in the absence of *dcn1*$^+$, we generated a strain containing the tagged allele of *pcu1*$^+$ and deleted for *dcn1*$^+$ (Δ*dcn1*), and observed a total loss of detectable neddylation of Pcu1p (Fig. 2A). This suggested that either neddylation of Pcu1p was not essential, or that the level was below detection by Western blot analysis. Deletion of *csn5*$^+$, in the *pcu1–13myc* strain lacking *dcn1*$^+$ (Δ*csn5*Δ*dcn1*) shifted the dynamic of Pcu1p neddylation to levels again detectable by Western blot analysis (Fig. 2A) demonstrating that Dcn1p is not essential for cullin neddylation. To investigate whether this reduction of Pcu1p neddylation had any effect on the steady-state levels of a known CRL1 substrate, a plasmid expressing FLAG-tagged *rum1*$^+$ was integrated into both wild-type and Δ*dcn1* cells. Expression was allowed to proceed for 18 hours in the absence of thiamine. Total cell extracts were subsequently prepared under denaturing conditions followed by immunoblotting with anti-FLAG antibody. The deletion of *dcn1*$^+$, and subsequent reduction in Pcu1p neddylation, was associated with a detectable increase in the steady-state levels of Rum1p (Fig. 2B).

These results showed that levels of Pcu1p neddylation are greater in the *uba3–10* strain compared to that of the Δ*dcn1* strain (Fig. 2C). Neddylation of Pcu3p was likewise compromised in both *uba3–10* and Δ*dcn1*$^+$ backgrounds. Due to the lower levels of basal neddylation observed [20], we were not able to detect

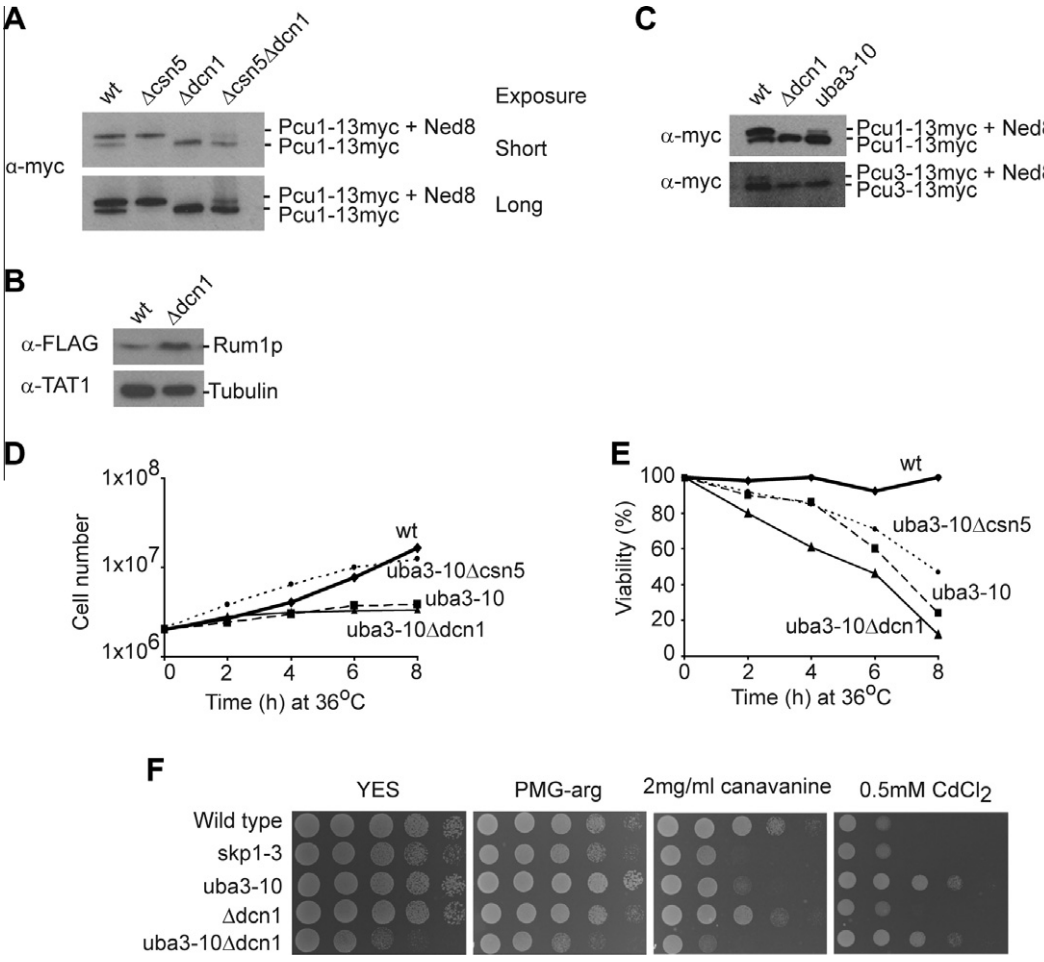

Fig. 2. Pcu1p neddylation levels do not determine UPS stress tolerance. (A) The tagged *pcu1–13myc* strain in the indicated genetic background were grown to log phase and lysate prepared under denaturing conditions and analysed by SDS-PAGE and immunoblotted with anti-myc antibody. (B) Wild-type and Δ*dcn1* cells expressing pDUAL-FFH1c *rum1*$^{+}$. Lysates were prepared under denaturing conditions and analysed by SDS-PAGE and immunoblotted with anti-FLAG antibody. (C) As in (A), but immunoblotting was preformed with anti-myc antibodies. (D) Wild-type *uba3–10*, *uba3–10Δcsn5*, *uba3–10Δdcn*1 yeasts were grown to early log phase in YES and shifted to 36 °C, and the cell number was determined at the indicated times. (E) As (D), but at each time point, 2 × 200 cells were plated onto YES medium and incubated at 25 °C for 5–7 days. Colonies on each plate were counted and the percentage of viable cells was calculated by normalisation to the colony number of the 0-h samples. (F) Fivefold serial dilutions of wild-type *uba3–10*, *skp1–3*, Δ*dcn1*, *uba3–10Δdcn1* yeasts were spotted onto YES, PMG-arg, PMG containing 2 μg/ml L-canavanine, and YES containing 0.5 mM cadmium chloride plates and incubated at the indicated temperatures for 3–7 days.

modification in either background (Fig. 2C). Thus, *dcn1*$^{+}$ is likely to the Ned8p ligase for all *S. pombe* cullins. To investigate whether a further reduction of Pcu1p neddylation would be viable, we crossed *uba3–10* with the Δ*dcn1* strain. As levels of Pcu1p neddylation were already undetectable in the Δ*dcn1* background, this was based on the hypothesis that some modification was still occurring. Remarkably the resulting strain was viable; suggesting that a very low level of neddylation was sufficient for viability. We reasoned that due to the further reduction of neddylation in the *uba3–10Δdcn1* strain, viability at the restrictive temperature should by lower compared to that of *uba3–10*. To test this, cultures of early log phase growing *uba3–10* and *uba3–10Δdcn1* yeasts were shifted from the permissive to the restrictive temperature and sampled every two hours (Fig. 2D and E). Consistent with the view that Pcu1p neddylation is essential, the *uba3–10Δdcn1* strains viability was further reduced, compared to the *uba3–10* strain, over the time course at the restrictive temperature. This hypothesis was further supported by the observation that the *uba3–10Δcsn5* strain, which would retain a higher basal Pcu1p neddylation in the absence of the metalloprotease activity (Fig. 2A), maintained greater viability over the time course compared to the *uba3–10* strain. These results suggest that loss of cullin neddylation, and subsequent SCF/CRL activity, is the first essential checkpoint encountered by impairment of Ned8p conjugation in the *uba3–10* strains.

To further investigate how the varying levels of Pcu1p neddylation, and the consequent effect on SCF/CRL activity, the strains were challenged with ubiquitin proteasome system (UPS) stresses. As such strains were grown on plates containing the known UPS challenging chemicals L-canavanine and cadmium chloride [21,22]. Surprisingly the *uba3–10* strain but not the Δ*dcn1* strain showed sensitivity to these stresses. This was not altered by the further reduction in Pcu1p neddylation, as the *uba3–10Δdcn1* showed comparable levels of tolerance (Fig. 2F). Wild-type, *skp1–3*, and Δ*dcn1* strains all showed sensitivity to 0.5 mM cadmium chloride, whilst the *uba3–10* and *uba3–10Δdcn1* strains showed increased tolerance (Fig. 2F). These results showed that the Δ*dcn1* strain had a functional SCF/CRL1 complex, even under conditions that would challenge the SCF/CRL1 ligase and require its efficient activity, despite the low levels of Pcu1p neddylation.

3.5. Generation and phenotypic analysis of *pcu1–2*

To further elucidate the requirements of the SCF/CRL1 ligase for proteasome stress we generated a conditionally lethal ts mutant of *pcu1*$^{+}$ (*pcu1–2*) (Fig. 3A). The mutation sites within *pcu1* (Table 3)

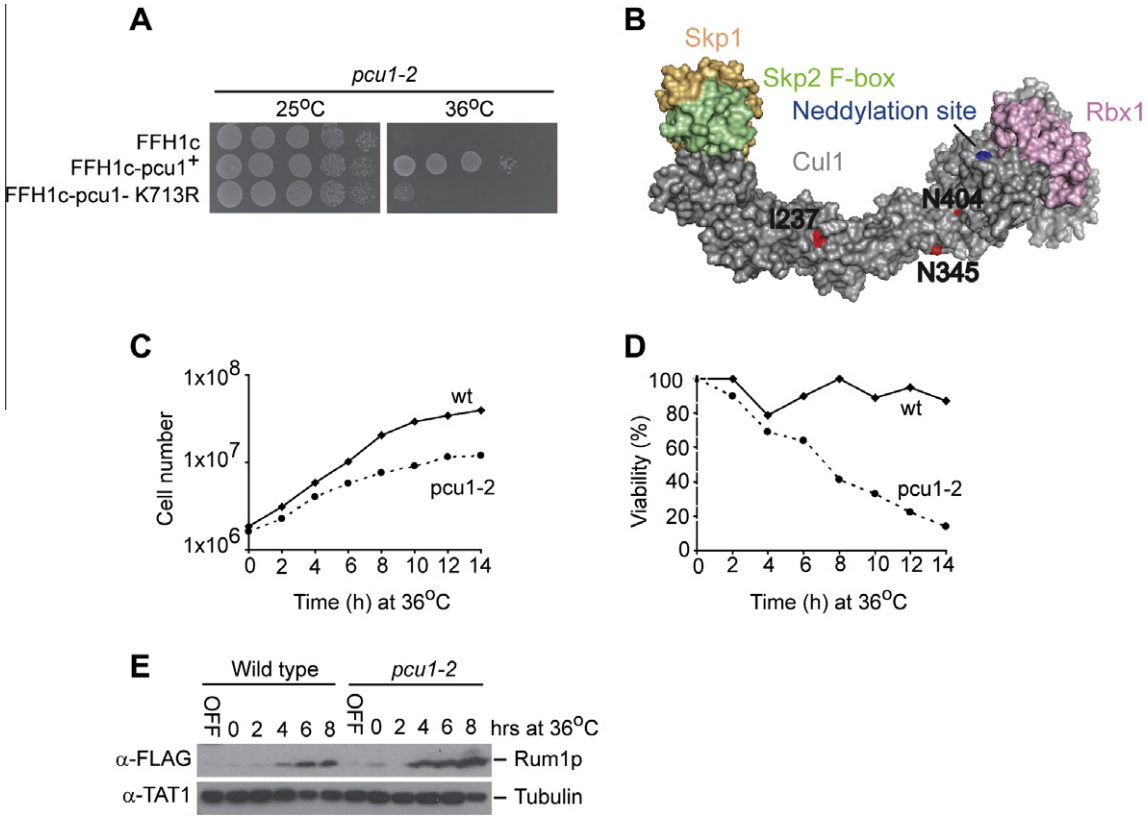

Fig. 3. *pcu1–2* cells are temperature-sensitive. (A) Stepwise fivefold serial dilutions of *pcu1–2* yeast integrated with the indicated plasmid were grown on PMG-Ura plates at the indicated temperature for 3–5 days (initial spots contained 1×10^5 cells). (B) Following sequence alignment the equivalent amino acids found mutated in *S. pombe pcu1–2* were identified in the human Cullin1 protein, and were mapped onto the solved crystal structure of the CUL1–RBX1–SKP2 complex. Images were made using MacPyMOL with (PDB:1LDK). Mutated residues are indicated in red. (C) Wild-type or *pcu1–2* yeast was grown in YES and shifted to 36 °C, and the cell number was determined at the indicated times. (D) As (C), but at each time point, 2×200 cells were plated onto YES medium and incubated at 25 °C for 5–7 days. Colonies on each plate were counted and the percentage of viable cells was calculated by normalisation to the colony number of the 0-h samples. (E) Time-course of wild-type and *pcu1–2* cells expressing pDUAL-FFH1c *rum1*$^+$ was shifted to the restrictive temperature for the indicated times. Lysates were prepared under denaturing conditions and analysed by SDS-PAGE and immunoblotted with anti-FLAG antibody.

Table 3
Mutation sites of pcu1 ts mutant strain.

Allele	Mutation sites
pcu1–2	I230S, R339G, N401D

were mapped onto the known crystal structure of the CUL1–RBX1–SKP1–Fbox^{SKP2} ligase complex following sequence alignment [23] (Fig. 3B). The mutated residues were found to be located in the cullin repeat domains of Pcu1p, with two of the residues highly conserved in both Pcu1p orthologues and paralogues, and possessing some degree of solvent accessibility. To confirm that the temperature sensitivity of the *pcu1–2* strain was solely due to the mutation of *pcu1*$^+$, we crossed the *pcu1–2* strain to its opposite wild-type mating type and showed that the temperature sensitivity segregated 2ts$^+$:2ts$^-$ (data not shown) and that the ts phenotype could be rescued by the exogenous expression of the *pcu1*$^+$ gene and not by the neddylation deficient Pcu1p^{K713R} mutant (Fig. 3A). Cultures of early log phase growing *pcu1–2* and wild-type cells were shifted from the permissive to the restrictive temperature and sampled every two hours. Cell number was determined by counting of cells on a Coulter counter (Fig. 3C). The viability of the *pcu1–2* strain was also determined (see Section 2) (Fig. 3D). These results showed that the lysine residue modified by Ned8p, in agreement with previous reports, is essential for Pcu1p activity and *S. pombe* viability.

3.6. Characterisation of the pcu1–2 mutant phenotype

To confirm that the *pcu1–2* mutant cells would effect the stabilization of a known SCF/CRL1 substrate, a plasmid expressing FLAG-tagged *rum1*$^+$, was integrated into wild-type and *pcu1–2* cells. Expression was allowed to proceed for 18 hours in the absence of thiamine, prior to cultures being shifted from the permissive to the restrictive temperature. Samples were taken every two hours for eight hours, and total extracts were prepared under denaturing conditions followed by immunoblotting with anti-FLAG. Inactivation of the SCF/CRL1 complex in the *pcu1–2* strain resulted in a time dependent stabilization of Rum1p (Fig. 3E).

3.7. Generation of a Pcu1p mutant that functions independently of neddylation

To investigate whether the reported in vitro independence of cullin neddylation, on CRL1 activity [7] could be recapitulated in vivo, we generated Pcu1p C-terminal WHB/ECTD truncation mutants. Previous reports had indicated that disruption of this domain would prevent cullin neddylation, both in vivo and in vitro [7,24,25]. Plasmids expressing Pcu1p C-terminal truncation mutants were integrated into the *pcu1–2* and *uba3–10* mutant strains, and their ability to rescue lethality at 36 °C was investigated. We observed that expression of Pculp$^{\Delta 720-767}$ and Pculp$^{\Delta 720-767/K713R}$ was able to rescue the lethality of the *pcu1–2* at the restrictive temperature (Fig. 4). These results indicate that in the absence of the entire Pcu1p

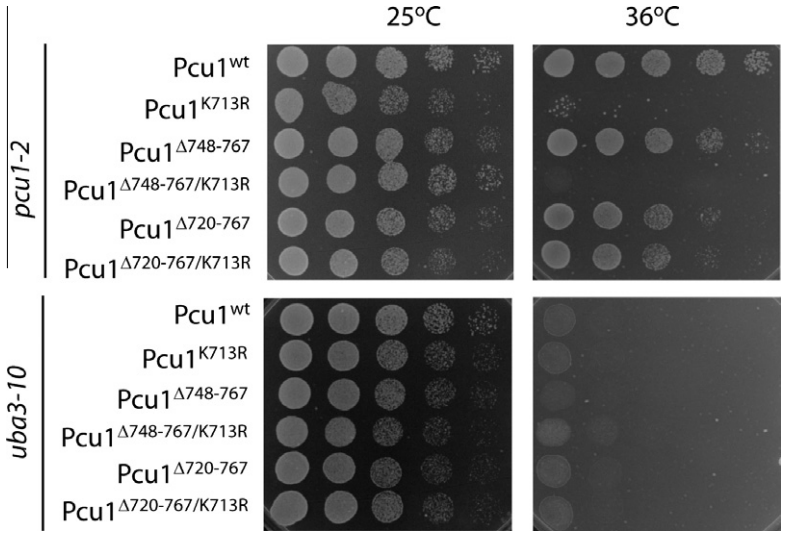

Fig. 4. Deletion of Pcu1p's C-terminal autoinhibitory domain negates the requirement for neddylation. (A) The *pcu1–2* mutant strain was transformed with plasmids expressing wild-type Pcu1, Pcu1^{K713R}, Pcu1$^{\Delta748-767}$, Pcu1$^{\Delta748-767/K713R}$, Pcu1$^{\Delta720-767}$, and Pcu1$^{\Delta720-767/K713R}$. Subsequently, stepwise fivefold serial dilutions of *pcu1–2* yeast integrated with the indicated plasmid were grown on PMG-Ura-Leu plates at the indicated temperature for 3 days (initial spots contained 1×10^5 cells). (B) As in (A), but the indicated Pcu1 constructs were transformed into the *uba3–10* mutant strain.

ECTD/WHB autoinhibitory domain, neddylation of Pcu1p is no longer required for CRL1 activity in vivo. Importantly, the expression of these Pcu1p mutants failed to complement the *uba3–10* allele (Fig. 4). We suggest therefore, that Ned8p has additional, essential roles out-with Pcu1p modification.

4. Discussion

NEDD8 has emerged as an important regulator of CRL mediated protein degradation, and reduction of cullin neddylation, via the activity of an AMP mimetic MLN4924, results in inhibition of approximately 20% of all proteasome-dependent protein degradation and apoptosis [26]. Surprisingly in this context, deletion of *dcn1*$^+$ in yeast, which resulted in undetectable levels of Pcu1p neddylation, maintained viability. As the *uba3–10* cells possess greater steady-state levels of Pcu1p neddylation compared to the Δ*dcn1* strain, and displayed distinct phenotypes to proteasome stresses; and as Pcu1p$^{\Delta720-767/K713R}$ can rescue the temperature sensitivity of the *pcu1–2* allele but not the *uba3–10* allele at the non-permissive temperature, suggests that it is likely that other essential functions of neddylation exist, via modification of undetermined substrates. We cannot rule out the intriguing possibility that Ned8p controls protein stability out-with modulation of the canonical CRL complex, given the differing sensitivities of the *uba3–10* and Δ*dcn1* strains to UPS stresses [27]. However, an alternative explanation would be that other components of the CRL complex are also neddylated, and this modification is reduced in the *uba3–10* strain, and not by deletion of the cullin specific ligase, Dcn1p. Indeed, Lag2p, the CAND1 budding yeast orthologue, is reported as being neddylated [28].

Although the full significance of neddylation as a regulatory mechanism has yet to be determined, it remains clear that the regulation of CRL activity represents a fundamental facet of this pathway. Importantly, our studies provide genetic evidence that Ned8p functions pleiotropically in fission yeast, although these elusive substrates remain to be determined.

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